Comparative Genomics of Two Closely Related Unicellular Thermo-Acidophilic Red Algae, *Galdieria sulphuraria* and *Cyanidioschyzon merolae*, Reveals the Molecular Basis of the Metabolic Flexibility of *Galdieria sulphuraria* and Significant Differences in Carbohydrate Metabolism of Both Algae[1]

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Unicellular algae serve as models for the study and discovery of metabolic pathways, for the functional dissection of cell biological processes such as organellar division and cell motility, and for the identification of novel genes and gene functions. The recent completion of several algal genome sequences and expressed sequence tag collections and the establishment of nuclear and organellar transformation methods has opened the way for functional genomics approaches using algal model systems. The thermo-acidophilic unicellular red alga *Galdieria sulphuraria* represents a particularly interesting species for a genomics approach owing to its extraordinary metabolic versatility such as heterotrophic and mixotrophic growth on more than 50 different carbon sources and its adaptation to hot acidic environments. However, the ab initio prediction of genes required for unknown metabolic pathways from genome sequences is not trivial. A compelling strategy for gene identification is the comparison of similarly sized genomes of related organisms with different physiologies. Using this approach, candidate genes were identified that are critical to the metabolic versatility of Galdieria. Expressed sequence tags and high-throughput genomic sequence reads covering >70% of the *G. sulphuraria* genome were compared to the genome of the unicellular, obligate photoautotrophic red alga *Cyanidioschyzon merolae*. More than 30% of the Galdieria sequences did not relate to any of the Cyanidioschyzon genes. A closer inspection of these sequences revealed a large number of membrane transporters and enzymes of carbohydrate metabolism that are unique to Galdieria. Based on these data, it is proposed that genes involved in the uptake of reduced carbon compounds and enzymes involved in their metabolism are crucial to the metabolic flexibility of *G. sulphuraria*.

Cyanidiales are small, unicellular, evolutionary, anciently diverged red algae that exist in many parts of the world in hot acidic habitats, both natural and man-made. Within this group that consists of the three genera Cyanidioschyzon, Cyanidium, and Galdieria the species delimitations have been frequently revised and updated. Currently, six species (*Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria maxima*, *Galdieria partita*, *Galdieria daedala*, and *Galdieria sulphuraria*) are recognized that belong to at least four distinct lineages (Ciniglia et al., 2004). The biology of Cyanidiales is interesting because, unlike most other eukaryotes, these organisms have adapted to extreme environments such as hot acidic springs and volcanic calderas and thus exhibit a number of quite unusual physiological traits, such as the resistance to a large array of toxic metal ions (Yoshimura et al., 1999, 2000; Nagasaka et al., 2002, 2004) and other stressors.

The phylogenetic position of the Cyanidiales has recently received increased attention. Yoon et al. (2002, 2004) have shown that the Cyanidiales form an ancient monophyletic group within the red algae (Yoon et al., 2002, 2004). These findings are supported by detailed studies on the evolution of starch metabolism in apicomplexa, rhodophytes, and chlorophytes (Coppin et al., 2005).

Cyanidioschyzon, Cyanidium, and Galdieria all occupy habitats with pH values between 0.05 and 3 and temperatures not above 56°C. These extreme conditions have put the Cyanidiales under high

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selective pressure over a long period of time and thereby minimized their physiological and morphological diversity, despite an early divergence of these genera as suggested from a comparison of 18S rRNA sequences (Gross et al., 2001; Yoon et al., 2002). Nevertheless, especially *C. merolae* and *G. sulphuraria* have developed a number of prominent differences (Gross, 1999; Albertano et al., 2000) that make a comparison of these two species particularly exciting. For example, *G. sulphuraria* propagates by endospores, whereas *C. merolae* divides by binary fission, and *C. merolae* lacks a cell wall and vacuole. Most interesting to the biochemist, however, is the extraordinary metabolic versatility of *G. sulphuraria* in comparison to *C. merolae*. Whereas *C. merolae* is confined to obligate photoautotrophic growth, a feature that is ascribed to rhodophytes in general (Raven et al., 1990), *G. sulphuraria* is able to grow photoautotrophically, heterotrophically, and mixotrophically. It thrives on more than 50 different carbon sources such as sugars, sugar alcohols, tricarboxylic-acid-cycle intermediates, and amino acids (Rigano et al., 1976, 1977; Gross and Schnarrenberger, 1995a; Gross, 1999; Oesterhelt et al., 1999), a metabolic flexibility matched by few other organisms.

Similar to the green alga *Chlamydomonas reinhardtii* (Rochaix, 1995, 2002; Dutcher, 2000; Harris, 2001; Hicks et al., 2001; Werner, 2002), unicellular red algae of the Cyanidiales have emerged as excellent model systems for the study of plant cell biology and metabolism. For example, the plastid division ring has first been discovered in *C. caldarium* RK-1, and the mitochondrial dividing apparatus was first described in *C. merolae* (for review, see Kuroiwa et al., 1998; Miyagishima et al., 2003).

Recent advances in the field of genome sequencing allowed new insights into the biology of these ancient and enigmatic algae at the molecular level. The plastid genome sequences of *C. merolae* and *C. caldarium* RK-1 (Glöckner et al., 2000; Ohta et al., 2003) and the mitochondrial genome of *C. merolae* (Ohta et al., 1998) have been sequenced. More recently, the genome sequence of *C. merolae* was determined (Matsuzaki et al., 2004), and a relatively large expressed sequence tag (EST) dataset from *G. sulphuraria* became available (Weber et al., 2004).

Cyanidiales have relatively small genomes in comparison to other eukaryotes: 17 Mb for *C. merolae* (Matsuzaki et al., 2004) and between 10 and 16 Mb for *Galdieria* spp. (Moreira et al., 1994; Muravenko et al., 2001). In addition, the almost complete absence of introns in genes of *C. merolae* (Matsuzaki et al., 2004) and the relatively low degree of genetic redundancy further simplify genome annotation and gene prediction. Moreover, the high portion of coding sequences (45%) in the *C. merolae* genome (Matsuzaki et al., 2004) makes the identification of regulatory elements relatively straightforward in comparison to larger and more redundant genomes. Finally, a recent report has demonstrated that *C. merolae* is amenable to transformation by electroporation, and evidence for a relatively high rate of homologous recombination was provided (Minoda et al., 2004), suggesting this unicellular alga is suitable for targeted gene-knockout approaches.

Comparative genomics (i.e. the comparison of genomes of different but related organisms) is a powerful tool to unravel the molecular foundations of observable traits and phenotypes that cannot easily be deduced from the analysis of individual genome sequences. Its core hypothesis is that the conserved regions of DNA between two species often encode for the common features of the organisms, while different traits will appear as differences in the genetic makeup between the two species (Hardison, 2003). The very similar genomes, such as those of Galdieria and Cyanidioschyzon, are then particularly useful for elucidating the key differences that account for the structural and metabolic differences in these organisms. Using the recently published genome sequence of *C. merolae* (Matsuzaki et al., 2004) and a large *G. sulphuraria* genomic dataset consisting of an EST collection (Weber et al., 2004) and 8 Mb of nonredundant genomic sequence (approximately 70% genome coverage; http://genomics.msu.edu/galdieria), we have initiated a comparative genomics approach to identify those genes of *G. sulphuraria* that might be crucial to its extraordinary metabolic flexibility. To this end, we set out to identify those Galdieria sequences that do not match any of the *C. merolae* genes and that display similarity to genes of known function from other organisms.

RESULTS

Sequence Data Used and Statistics of Genomic Comparisons

The genome sequence of *C. merolae* was published recently (Matsuzaki et al., 2004), and all sequence data can be downloaded from the *Cyanidioschyzon merolae* Genome Project Web site (http://merolae.biol.s.u-tokyo.ac.jp/). The autotrophic and heterotrophic *G. sulphuraria* cDNA libraries used in this comparative approach have recently been described (Weber et al., 2004). The EST dataset consists of 3,099 clustered ESTs and covers approximately 30% of the genome. A BLASTN search of the ESTs against the *C. merolae* genomic sequence database revealed 2,644 significant hits (i.e. bit score >50 and $E < e^{-5}$), and some of the genomic sequences matched more than one EST. Thirty megabases of high-throughput genomic sequencing reads from the *G. sulphuraria* genome sequencing project were assembled into contigs, yielding 8 Mb of nonredundant genomic sequence that cover approximately 70% of the Galdieria genome, and compared to the *C. merolae* genome and the Galdieria EST dataset. Approximately 20% of the contigs matched at least 1 of the 3,099 ESTs. Contigs

were matched by BLASTX against the Cyanidioschyzon and the nonreduntant database. Close to 30% did not match any of the Cyanidioschyzon and nonreduntant protein sequences. All Galdieria EST and genomic data (contigs and genomic reads) can be downloaded from the *Galdieria sulphuraria* Genome Project Web site (http://genomics.msu.edu/galdieria).

Galdieria Genes Have More Introns Than Those of Cyanidioschyzon

A comparison of the *G. sulphuraria* contigs to the EST dataset revealed that approximately 50% of all tested Galdieria genes seem to contain introns. Based on 10 randomly selected genes containing 1 to 3 introns, intron lengths of 45 to 65 bases were found. The borders of the introns displayed typical spliceosomal features as previously described for a light-harvesting complex gene from *G. sulphuraria* (Marquardt et al., 2000); in 80% of the cases, the splice donor site started with the sequence GU and the same percentage was observed for the splice acceptor site, which consisted of AG. Nevertheless, in 50% of the cases the branching site was composed of the consensus sequence CUPuAPy located 15 to 30 bases upstream of the acceptor site. In *C. merolae*, only 26 genes containing introns were found (Matsuzaki et al., 2004). Although this point needs further investigation, it appears that *G. sulphuraria* genes contain significantly more introns than the corresponding *C. merolae* genes.

Carbohydrate Metabolism in *G. sulphuraria* and *C. merolae*

Galdieria is the only member of the Cyanidiales that is capable of heterotrophic growth. To introduce different sugars into the central carbohydrate metabolism, cells need a broad variety of sugar kinases. The *G. sulphuraria* genome encodes putative gluco-, galacto-, fructo-, glycero-, xylulo-, and ribokinases. All of them exhibit closest similarity to prokaryotic enzymes and not to the corresponding plant sugar kinases. Measurements of purified fructokinase from *G. sulphuraria* had previously shown that the substrate specificity of the enzyme was similar to bacterial orthologs (Fru and Man) and not to that of hexokinases from green plants (Glc, Fru, and Man; Heilmann et al., 1997). Despite being an obligate photoautotroph, *C. merolae* has a genome that harbors the same spectrum of sugar kinases as *G. sulphuraria*, which is capable of heterotrophic growth. *G. sulphuraria* encodes a number of polyol dehydrogenases, which are required for introducing sugar alcohols into its heterotrophic metabolism (Table I). The occurrence of a sorbitol- and a xylitol-dehydrogenase, as previously reported based on biochemical evidence (Stein et al., 1997), could now be verified by genomic data. Judged from its genome, *C. merolae* exhibits a similar enzymatic makeup as *G. sulphuraria*.

Man metabolism is not very extensive in most plants. Phosphorylation of this sugar often represents a dead end and leads to phosphate depletion of cells (Herold and Lewis, 1977). For *G. sulphuraria*, however, it has previously been shown that Man can be efficiently introduced into the metabolism by phosphorylation, intramolecular transfer of phosphate groups, and isomerization (Oesterhelt et al., 1996; Heilmann et al., 1997). Even though *C. merolae* cannot grow heterotrophically and free Man should therefore not occur in the alga, a pathway for Man metabolism does exist as both phosphomannomutase and Man-P isomerase are present in the genome.

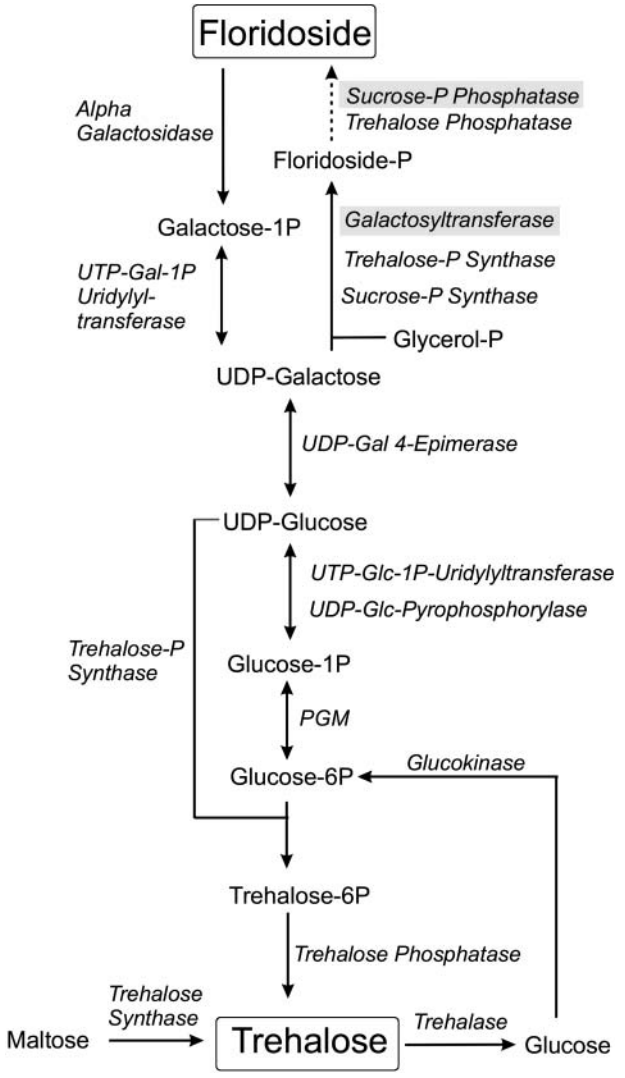

Figure 1. Metabolism of floridoside and trehalose in *G. sulphuraria* and *C. merolae*. Enzymes involved in the metabolism of floridoside and trehalose were derived from genomic reads and EST sequences. Those enzymes found exclusively in *G. sulphuraria* and not in *C. merolae* are shaded in gray. The enzymatic setup of trehalose metabolism is complete in both Cyanidiales. Genes encoding enzymes for the metabolism of floridoside were identified only in *G. sulphuraria*. The involvement of a Suc-P phosphorylase-like protein in floridoside synthesis is hypothetical at this point. Gene accession numbers are given in Table I.

Another sugar that many plants cannot efficiently utilize is Gal. It often accumulates as Gal-1P or as UDP-Gal (Roberts et al., 1971). In contrast to many higher plants, however, both Cyanidiales genomes encode enzymes for a complete Gal metabolism. We found a putative galactokinase, a UTP-Gal-1P-uridylyltransferase as well as UDP-Gal 4-epimerase (Figs. 1 and 2). A gene with similarity to UDP-Gal pyrophosphorylase could not be detected, indicating that the Cyanidiales use the Leloir pathway for Gal metabolism and not the Isselbacher pathway, a feature that is usually attributed to bacteria and animals but not to plants. Biochemical studies on activities of Gal-metabolising enzymes in *G. sulphuraria*, however, support the existence of the Leloir pathway in Galdieria (Gross and Schnarrenberger, 1995b; Prosselkov et al., 1996).

Overall, *G. sulphuraria* and *C. merolae* exhibit a strikingly similar enzymatic makeup of sugar and polyol metabolism. The inability of the Cyanidioschyzon to use externally supplied carbon sources as substrates for heterotrophic growth can therefore not be attributed to a lack of essential enzymes of carbon metabolism.

The Large Number of Putative Carbohydrate Transporters Encoded by the Genome of *G. sulphuraria* Is a Key Difference to *C. merolae*

A major physiological distinction between *C. merolae* and *G. sulphuraria* is Galdieria's ability to grow mixotrophically and heterotrophically, whereas Cyanidioschyzon is an obligate-photoautotrophic organism. Nevertheless, we found that the *C. merolae* genome encodes all the enzymes for the metabolism of, for example, Glc, Man, and Gal. However, a crucial prerequisite for heterotrophic growth is the existence of uptake systems for exogenous substrates. Our initial analysis of the Galdieria EST dataset indicated that the genome of this alga encodes a very high number of putative monosaccharide transporters, whereas the genome of Cyanidioschyzon encodes only a single

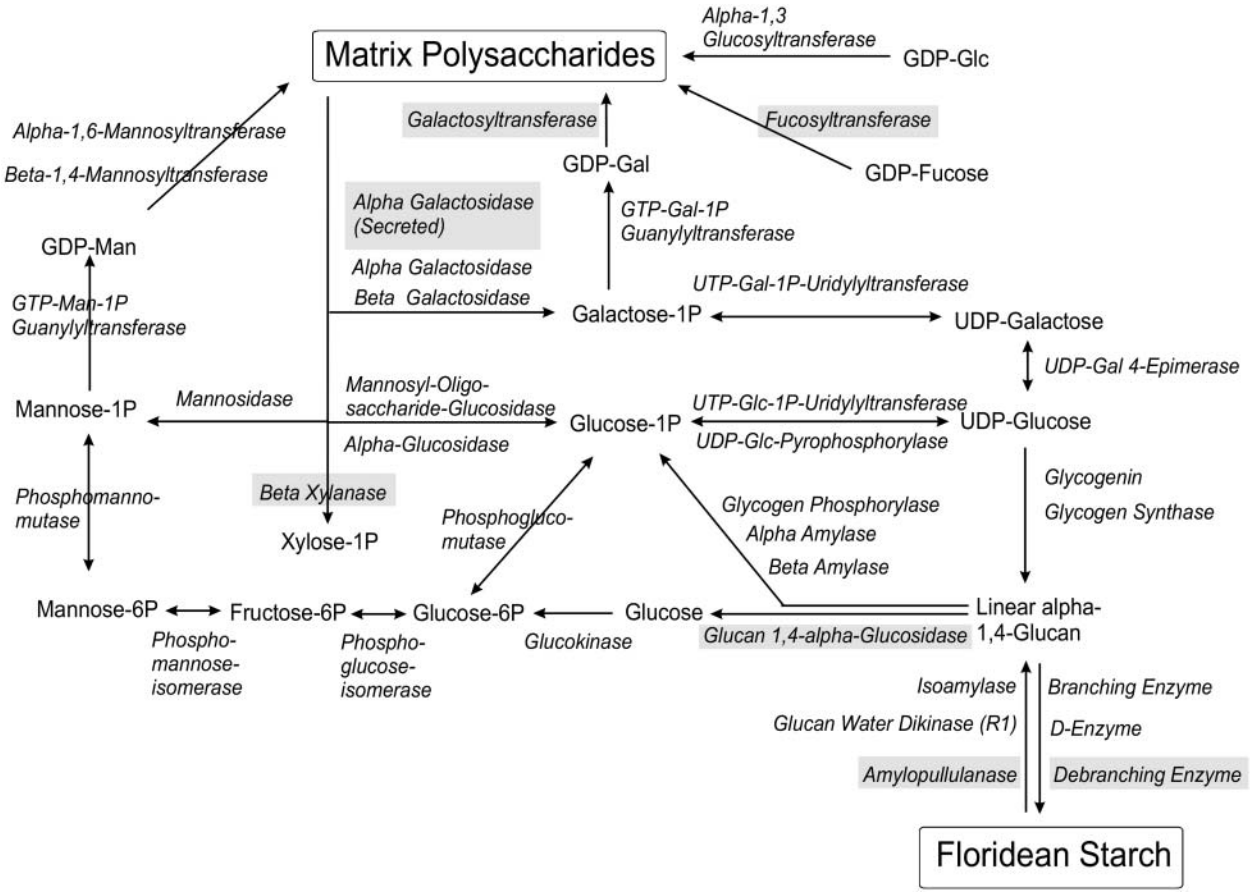

Figure 2. Metabolism of storage glucans and matrix polysaccharides in *G. sulphuraria* and *C. merolae*. Enzymes involved in polysaccharide metabolism were derived from genomic reads and EST sequences. Those enzymes found exclusively in *G. sulphuraria* and not in *C. merolae* are shaded in gray. They are mainly involved in cell wall and matrix polysaccharide metabolism. The difference between floridean starch in *G. sulphuraria* and the more highly branched phytoglycogen in *C. merolae* is reflected by the absence of debranching enzyme in the latter. Also, the breakdown of storage glucan is more diverse in *G. sulphuraria*. Gene accession numbers are given in Table I.

putative Ara transporter (Weber et al., 2004). A more careful analysis of the EST dataset and a survey of the genomic sequence reads corroborate this initial observation. In addition to the putative Glc transporters described previously (Weber et al., 2004), the Galdieria genome encodes at least four different Gal proton symporters, four independent Fru proton symporters, and one Ara proton symporter. Moreover, we could identify several putative Suc proton symporters and a number of ESTs and genomic sequences encoding at least eight distinct putative sodium *myo*-inositol symporters (Fig. 3). In total, we have identified 28 distinct sugar transporter genes in *G. sulphuraria* that do not have an equivalent in the genome of *C. merolae* (Table II). Although it is difficult to predict the subcellular localization of these transporters by in silico analysis only, it is reasonable to assume that many of these transporters are targeted to the plasma membrane. Hence, Galdieria appears to have a much larger repertoire of carbohydrate transporters in its plasma membrane than Cyanidioschyzon. The associated ability to take up a large variety of sugars from the environment, as demonstrated previously in physiological studies (Oesterhelt et al., 1999; Oesterhelt and Gross, 2002), is therefore most likely essential for the extraordinary metabolic flexibility of *G. sulphuraria*. Conversely, the inability of *C. merolae* to use supplied sugars is due to the absence of appropriate uptake systems.

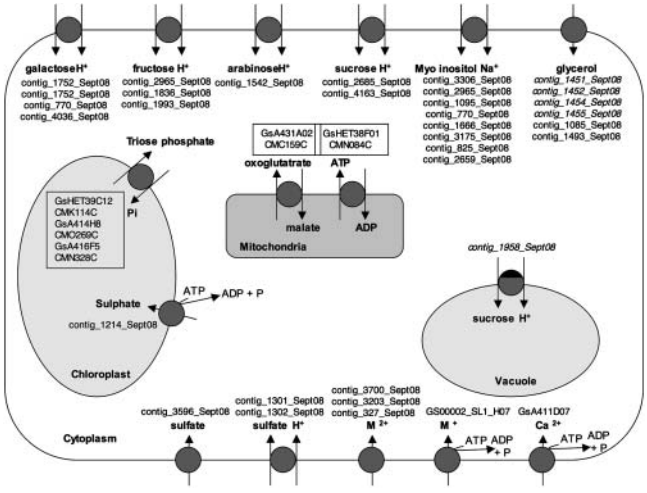

Figure 3. Solute transporters in *G. sulphuraria* and *C. merolae*. The metabolic flexibility of *G. sulphuraria* is reflected by a significantly higher number of solute transporters in comparison to *C. merolae*. With the exception of transporters of the plastid and mitochondrial membranes, only transporters that are exclusive to *G. sulphuraria* are depicted in this scheme. Sequence identifiers starting with "contig" are genomic sequences from *G. sulphuraria*. Sequence identifiers starting with "Gs" are ESTs from *G. sulphuraria*. Both contigs and ESTs sequences are available from the Galdieria Genome Project Web site. Sequence identifiers starting with "CM" are *C. merolae* genomic sequences and are accessible from the *C. merolae* genome project Web site. Boxes indicate common gene function. Clusters of sequence identifies given in italic are coding for same protein.

Aquaporin-Like Glycerol Permeases Potentially Enable Galdieria to Use Glycerol as a Carbon Source

The genomes of both Cyanidioschyzon and Galdieria encode the enzymes for glycerol metabolism, such as glycerol kinase. Again, the key difference between both algae is most likely Cyanidioschyzon's apparent inability to take up glycerol from the environment. The Galdieria genome contains at least four genes that encode putative glycerol permeases of the aquaporin type (de Groot and Grubmuller, 2001; Thomas et al., 2002; Stroud et al., 2003) that are not present in the *C. merolae* genome and that most likely enable *G. sulphuraria* to take up external glycerol to fuel its energy metabolism.

The *G. sulphuraria* Genome Harbors a Larger Number of Genes Encoding Cation and Anion Transporters

The *G. sulphuraria* genome harbors at least five cation transporters, which are absent from the genome of *C. merolae*. Two of these transporters share a high degree of sequence similarity with ATP-dependent cation transporters (ATP-binding cassette transporters), while the other three are annotated in the National Center for Biotechnology Information (NCBI) database as metal permeases. When compared against NCBI's nonredundant database, prokaryotic orthologs were identified as best matches for all five putative cation transporters. It is likely that some of these transporters are crucial to the extraordinary tolerance of Galdieria toward toxic metal ions. In addition, two putative sulfate transporters were exclusively identified in *G. sulphuraria* and not in *C. merolae*. As for most other transporters described in this manuscript, our functional assignments are based mainly on the annotation of related proteins in the NCBI database. Since many of these putative transporters have not been characterized yet, these transporters might have other functions than, e.g. sulfate transport.

Plastid and Mitochondrial Transporters

Plastid and mitochondrial transport processes are crucial to both Galdieria and Cyanidioschyzon, and essential differences between both organisms were therefore neither expected nor found. For the sake of brevity, only some representative examples such as the plastidic triose phosphate translocator and the mitochondrial ATP and dicarboxylate transporters are given in Figure 3. A plastidic ATP transporter, similar to those in green plants, has been described previously (Linka et al., 2003).

Biosynthesis and Breakdown of Polyglucans

Intracellular storage glucans are common to all living organisms. Chlorophyta as well as seed plants synthesize and store starch inside the plastid stroma and use ADP-Glc as precursor for chain elongation

Table I. *Predicted protein functions and sequence identifiers of genes encoding putative proteins of glucan metabolism in G. sulphuraria and C. merolae*

For *G. sulphuraria* the contigs as well as EST identifiers are given. All sequences can be retrieved from http://genomics.msu.edu/galdieria. When the number of estimated genes is higher than one, clusters of sequences given in italic belong to the same gene. –, No data.

Predicted Protein Function	*G. sulphuraria*			*C. merolae*
	Sequence Identifier	EST	Estimated No. of Gene	Sequence Identifier
Glucokinase	contig_1537_Sept08	–	1	gnl|CMER|CMO276C
Galactokinase	contig_2290_Sept08	–	1	gnl|CMER|CMN198C
	contig_2291_Sept08			
Fructokinase	contig_2780_Sept08	–	1	gnl|CMER|CMG166C
Xylulokinase/ribulokinase	contig_1564_Sept08	–	1	gnl|CMER|CMF097C
Ribokinase	contig_1660_Sept08	–	1	gnl|CMER|CMI123C
Glycerol kinase	contig_2175_Sept08	GS02230	1	gnl|CMER|CMJ173C
	contig_2177_Sept08	A4_40D08		
	contig_2177_Sept08			
	contig_4077_Sept08			
Phosphoglucomutase	*contig_3683_Sept08*	–	2	gnl|CMER|CMT285C
	contig_3540_Sept08			gnl|CMER|CMJ272C
	contig_2329_Sept08			
Phosphomannomutase	contig_1164_Sept08	A4_10D11	2	gnl|CMER|CMT314C
	contig_1257_Sept08			gnl|CMER|CMQ067C
Glc-6P-isomerase	*contig_2566_Sept08*	–	2	gnl|CMER|CMO124C
	contig_1629_Sept08			gnl|CMER|CMT497C
	contig_1635_Sept08			
Man-6P-isomerase	contig_3031_Sept08	–	1	gnl|CMER|CMQ359C
Sorbitol-dehydrogenase	contig_1925_Sept08	–	2	–
	contig_2510_Sept08			
Glycogenin-glucosyltransferases	contig_3301_Sept08	HET_19E06	1	gnl|CMER|CMG174C
	contig_3228_Sept08			
Glycogenin	contig_2667_Sept08	–	1	gnl|CMER|CMK020C
	contig_4140_Sept08			
Glycogen synthase	contig_2619_Sept08	HET_25C3	2	gnl|CMER|CMM317C
	contig_2433_Sept08			
4-α-Glucanotransferase D-enzyme	contig_3527_Sept08	–	–	gnl|CMER|CMP352C
				gnl|CMER|CMT204C
(1,4)-α-Glucan branching enzyme	GS00084_SR2_H07	–	1	gnl|CMER|CMH144C
	GS00015_SR2_D08			
Isoamylase type debranching enzyme	contig_1169_Sept08	–		–
Isoamylase	contig_1284_Sept08	A4_41H04		gnl|CMER|CMS197C
α-Amylase	contig_1343_Sept08	A4_6H07	3	gnl|CMER|CMO053C
	contig_3603_Sept08			gnl|CMER|CMB158C
	contig_1766_Sept08			gnl|CMER|CMD033C
β-Amylase	contig_2531_Sept08	A4_14E02	2	gnl|CMER|CMJ087C
	contig_2318_Sept08			
Glycogen phosphorylase	contig_2616_Sept08	A4_22A10|	1	gnl|CMER|CMD184C
	contig_2615_Sept08	HET_36G09|		
Amylopullulanase	contig_4051_Sept08	–	2	–
	contig_923_Sept08			
	contig_1343_Sept08			
Glucan (1,4)-α-glucosidase	contig_4152_Sept08	–	1	–
Fucosyltransferase	contig_1537_Sept08	–		–
Galactosyltransferase	contig_790_Sept08	–	1	–
	contig_727_Sept08			
Glucosyltransferase	contig_3280_Sept08	–	1	gnl|CMER|CMH028C
	contig_1917_Sept08			
Mannosyltransferase	contig_2161_Sept08	GS11580	4	gnl|CMER|CMR050C
	contig_1982_Sept08	A4_34E06		gnl|CMER|CMT636C
	contig_2830_Sept08			
	contig_3519_Sept08			
	contig_2441_Sept08			
Xylanase	contig_3042_Sept08	–	1	–

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Predicted Protein Function	*G. sulphuraria*			*C. merolae*
	Sequence Identifier	EST	Estimated No. of Gene	Sequence Identifier
Nucleoside-diphosphate-sugar epimerase	contig_2145_Sept08	HET_37D08	4	gnl|CMER|CMH105C
	contig_2038_Sept08			gnl|CMER|CMT520C
	contig_3239_Sept08			
	contig_1896_Sept08			
UDP-Glc 4-epimerase;	contig_2145_Sept08	A4_14C11	2	gnl|CMER|CMA041C
UDP-Gal-4-epimerase	contig_1514_Sept08			
UTP-Gal-1P-uridylyltransferase	contig_1031_Sept08	HET_36B03	1	gnl|CMER|CMQ030C
		HET_11G09		
UTP-Glc-1P-uridylyltransferase	contig_3254_Sept08	A4_36F11	–	gnl|CMER|CMS159C
α-Galactosidase	contig_2248_Sept08	HET_24G12/	4	gnl|CMER|CMG050C
	contig_4118_Sept08	A4_22C06		
	contig_2884_Sept08			
	contig_921_Sept08			
	contig_2883_Sept08			
Secreted α-galactosidase	contig_2309_Sept08	–	2	–
	contig_2248_Sept08			
	contig_4118_Sept08			
β-Galactosidase	contig_2366_Sept08	–	10	gnl|CMER|CMP078C
	contig_2927_Sept08			
	contig_3600_Sept08			
	contig_3575_Sept08			
	contig_2560_Sept08			
	contig_825_Sept08			
	contig_938_Sept08			
	contig_3599_Sept08			
	contig_1542_Sept08			
	contig_1260_Sept08			
	contig_1542_Sept08			
	contig_1541_Sept08			
α-Glucosidase	contig_1647_Sept08	A4_5D11	3	gnl|CMER|CMB095C
	contig_3264_Sept08	A4_32D09		
	contig_499_Sept08			
	contig_4032_Sept08			
	contig_814_Sept08			
Mannosyl-oligosaccharide glucosidase	*contig_1647_Sept08*	A4_6F10	1	gnl|CMER|CMF070C
	contig_4027_Sept08	A4_5D11		
β-Mannosidase	contig_2366_Sept08	–	1	–
APS-reductase	contig_4130_Sept08	–	1	gnl|CMER|CMT162C
PAPS-reductase	contig_3550_Sept08	–	1	gnl|CMER|CMS202C
Trehalose synthase	contig_993_Sept08	A4_6H07	1	gnl|CMER|CMB158C
	contig_1765_Sept08			
	contig_1766_Sept08			
	contig_1767_Sept08			
Trehalose-P synthase	*contig_1915_Sept08*	A4_23G07	6	gnl|CMER|CMI293C
	contig_1916_Sept08	A4_8G11		gnl|CMER|CMO053C
	contig_1206_Sept08	HET_40F04		gnl|CMER|CMP219C
	contig_0_Sept08	*A4_10B08*		
	contig_3603_Sept08	*A4_17F02*		
	contig_3672_Sept08	*HET_40F04*		
	contig_2238_Sept08	HET_42A08		
	contig_1411_Sept08	A4_14F07		
	contig_1410_Sept08			
Trehalose phosphatase	contig_3672_Sept08	HET_19A04	2	gnl|CMER|CMS178C
	contig_2824_Sept08			gnl|CMER|CMQ148C
Trehalase	contig_1104_Sept08	HET_20C04	1	gnl|CMER|CMH100C
	contig_1108_Sept08			gnl|CMER|CMN097C

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Predicted Protein Function	*G. sulphuraria*			*C. merolae*
	Sequence Identifier	EST	Estimated No. of Gene	Sequence Identifier
Suc-P synthase	contig_2706_Sept08 contig_2707_Sept08	HET_13E07 HET_24A9 A4_11B10 A4_T7comboE05 A4_T7comboC01 A4_11E07 A4_32F05	1	gnl|CMER|CMG051C
Suc-P phosphatase	contig_1792_Sept08	–	1	–

(Ball et al., 1996; Smith et al., 1997; Smith, 1999). In contrast, starch synthesis in red algae (i.e. floridean starch biosynthesis) is confined to the cytosol. It differs from that of the green plant lineage because UDP-Glc serves as the principle precursor and because no amylose is present in floridean starch (Meeuse and Kreger, 1954; Meeuse, 1960; Nagashima et al., 1971; Viola et al., 2001). While earlier studies indicated that *C. merolae* would contain phytoglycogen, whereas *G. sulphuraria* would use floridean starch as storage glucan (Seckbach, 1994), recent analysis revealed that both algae synthesize semicrystalline floridean starch (Coppin et al., 2005). Glucan metabolism of the Cyanidiales therefore resembles that of other red algae (Viola et al., 2001; Nyvall Collén et al., 2004).

As outlined below, our genomic comparison of *C. merolae* and *G. sulphuraria* revealed a complete set of candidate genes required for the biosynthesis and breakdown of storage glucans. Yet, we also detected distinct differences in the enzymatic makeup of the two Cyanidiales (Table I; Figs. 1 and 2). Both *G. sulphuraria* and *C. merolae* contain one gene encoding a putative glycogenin (UDP-Glc:glycogenin glucosyltransferase; EC 2.4.1.186; Table I). In metazoans, this enzyme serves as primer for the consecutive-chain elongation during glycogen synthesis. It has glucosyltransferase activity and glycosylates itself (Lomako et al., 1988, 2004). Sequence comparison against the nonredundant NCBI database also showed similarity of the Cyanidiales glycogenins to putative glycogenins from Arabidopsis (*Arabidopsis thaliana*). However, a functional plant glycogenin has not been demonstrated yet, and this protein might serve other functions in seed plants.

After the initiation of glucan synthesis, chain elongation in *C. merolae* and *G. sulphuraria* proceeds via glycogen (starch) synthase (EC 2.4.1.11) homologs using UDP-Glc. Modifications of the linear α-(1,4)-glucan and branch points are introduced by the D-enzyme, the branching enzyme, and isoamylase.

The breakdown of branched storage glucans is most likely accomplished by amylases in cooperation with glucan water dikinase (R1), which phosphorylates starch prior to degradation by amylases (Yu et al., 2001; Ritte et al., 2002). R1 could be identified in Cyanidioschyzon but not in the Galdieria genome. However, using an antibody directed against R1 from potato (*Solanum tuberosum*), we could detect an immunoreactive protein in crude extracts of autotrophically grown *G. sulphuraria* (data not shown). The presence of R1 in both Cyanidiales can thus be assumed. Linear α-(1,4)-glucan is probably broken down by α- and β-amylase as well as homologs of glycogen phosphorylase in both Cyanidales. In addition, *G. sulphuraria* also possesses an amylopullulanase that is able to catalyze the hydrolysis of both α-(1,6)- and α-(1,4)-glycosidic linkages. This enzyme has not previously been reported for plants, but only for bacteria (Mathupala et al., 1993; Ara et al., 1995; Chen et al., 2001). Moreover, *G. sulphuraria* possesses an additional glucan-(1,4)-α-glucosidase that releases terminal β-Glc units from the nonreducing ends of the glucan.

In summary, the glucan metabolism of Galdieria seems to be more advanced and diverse than that of Cyanidioschyzon (Fig. 2). Essential enzymes for the biosynthesis of semicrystalline floridean starch, however, are encoded in the genomes of both algae.

Candidate Genes for Cell Wall Biosynthesis in *G. sulphuraria*

In addition to cytosolic α-glucan, the cell wall of red algae represents a large sink for carbohydrates. Unlike *G. sulphuraria*, *C. merolae* is devoid of a cell wall and the genome of *C. merolae* should therefore lack many of the genes coding for enzymes involved in cell wall biosynthesis, modification, or degradation. Accordingly, we found several putative fucosyltransferases, galactosyltransferases, and xylanases exclusively in the *G. sulphuraria* genome (Table I). In addition, there are mannosyltransferases and glucosyltransferases that were also present in *C. merolae*. These enzymes are potentially involved in the biosynthesis of basic cell wall components of *G. sulphuraria* such as cellulose and hemicellulose as well as that of other heteroglucans associated with the cell wall, (e.g. xyloglucan, galactomannan, or glucomannan (Elbein, 1969; Hanna et al., 1991; Perrir et al., 1999; Rose and Bennett, 1999). Sugar precursors are usually introduced into the cell wall metabolism via their GTP-activated analogs (Seifert, 2004). Although this requires further inves-

Table II. *Predicted protein function and sequence identifiers of solute transporters exclusively found in G. sulphuraria*

All sequences can be retrieved from http://genomics.msu.edu/galdieria. When the number of estimated genes is higher than one, clusters of sequences given in italic belong to the same gene.

Predicted Protein Function	Sequence Identifier	EST	Estimated No. of Genes
Gal proton symporter	contig_1752_Sept08	–	2
	contig_1752_Sept08		
	contig_770_Sept08		
	contig_4036_Sept08		
Fru proton symporter	contig_2965_Sept08	–	3
	contig_1836_Sept08		
	contig_1993_Sept08		
Ara proton symporter	contig_1542_Sept08	–	1
Suc proton symporter	contig_2685_Sept08	–	2
	contig_4163_Sept08		
Myo-inositol Na^+ symporter	contig_3306_Sept08	HET_32G03	8
	contig_2965_Sept08	HET_18B07	
	contig_1095_Sept08	*GS02210*	
	contig_770_Sept08	*HET_2G7*	
	contig_1666_Sept08	A4_4E06	
	contig_3175_Sept08		
	contig_825_Sept08		
	contig_2659_Sept08		
Glycerol facilitator protein	*contig_1451_Sept08*	*GS04110*	3
	contig_1452_Sept08	*A4_8F04*	
	contig_1454_Sept08		
	contig_1455_Sept08		
	contig_1085_Sept08		
	contig_1493_Sept08		
Sulfate transporter	contig_3596_Sept08	–	1
Sulfate proton symporter	contig_1301_Sept08	–	1
	contig_1302_Sept08		
Divalent cation transporter	contig_3700_Sept08	*A4_34G04*	3
	contig_3203_Sept08	*A4_T7comboA07*	
	contig_327_Sept08		
Monovalent cation ATPase	GS00002_SL1_H07	–	1
Ca^{2+} ATPase	–	A4_11D07	1
Chloroplastic ATP-binding cassette-like sulfate transporter	contig_1214_Sept08	–	1
Vacuolar putative Suc proton symporter	contig_1958_Sept08	–	1

tigation, the large number of putative nucleoside-diphosphate-sugar-epimerases encoded by the genome of *G. sulphuraria* indicates a high degree of interconnectivity between the GDP-sugar pools and likely reflects the metabolic flexibility of *G. sulphuraria* in contrast to *C. merolae* (Fig. 2).

For the breakdown of heteroglucans, we found genes encoding α- and β-galactosidase as well as α-glucosidase, mannosyl-oligosaccharide glucosidase, and mannosidase in both Cyanidiales (Table I; Fig. 2).

Disaccharide Metabolism

Apart from complex polysaccharides, Cyanidiales contain important disaccharides, and the comparative genomics approach revealed a number of candidate genes that are potentially involved in the metabolism of disaccharides (Table I; Fig. 1).

Trehalose is a general anti-stress reagent (osmolyte) in yeast (*Saccharomyces cerevisiae*) and plants (Hounsa et al., 1998). In addition, a role in sugar signaling has been attributed to the trehalose biosynthesis intermediate trehalose-6-P (Goddijn and Smeekens, 1998; Schluepmann et al., 2003, 2004; van Dijken et al., 2004). All enzymes required for trehalose biosynthesis, that is trehalose-P synthase (α,α-trehalose-P synthase, UDP-forming; EC 2.4.1.15), trehalose-P (trehalose-6-P phosphohydrolase; EC 3.1.3.12), and trehalose synthase (Maltose α-D-glucosyltransferase; EC 5.4.99.16) were found in the *C. merolae* and *G. sulphuraria* genomes. The latter enzyme has not previously been reported from plants but has been extensively characterized in yeast (Londesborough and Vuorio, 1993; Bell et al., 1998). Furthermore, α-αtrehalase (α,α-trehalose glucohydrolase; EC 3.2.1.28), an enzyme involved in the

breakdown of trehalose, is present in both Cyanidiales. Phylogenetic analysis of *G. sulphuraria* gene products potentially involved in trehalose biosynthesis and breakdown supports the functional assignments reported above (data not shown).

While Suc is the main product of photosynthesis in many green plants, it does not seem to play a major role in red algae (Karsten et al., 1999). Therefore, it was surprising to find genes encoding a putative Suc-P synthase (UDP-Glc:D-Fru-6-P 2-α-D-glucosyltransferase; EC 2.4.1.14) in the *C. merolae* genome (Table I). Orthologous genes are also present in *G. sulphuraria*. However, the general sequence comparison also showed a number of similarities to glycosyltransferases, some of which are potentially involved in cell wall biogenesis. In addition, a Suc-6-P phosphorylase that is required for a complete Suc biosynthetic pathway (Lunn et al., 2000; Lunn and MacRae, 2003) is missing in the *C. merolae* genome. It is therefore unlikely that Suc plays a role in the alga. However, the *G. sulphuraria* genome encodes a putative Suc-6-P phosphorylase, which is also similar to Suc-phosphatases and a hydrolase of the haloacid dehalogenase superfamily (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR006357) in prokaryotes. Although enzymes of Suc metabolism from green plants show the highest similarity to these Cyanidiales proteins, the functional assignment remains uncertain, in particular because a functional Suc biosynthesis pathway in Cyanidales has not been demonstrated previously.

An alternative hypothesis would be that these enzymes are involved in floridoside biosynthesis (Fig. 1). Floridoside [α-D-galactopyranosyl-(1,2)-glycerol] represents the main sink for photosynthetic carbon in red algae, and like trehalose, it serves as an osmolyte and is accumulated to high amounts under stress conditions (Karsten et al., 1993). Within the Cyanidiales, floridoside seems to be confined to *G. sulphuraria* and *C. caldarium* (De Luca and Moretti, 1983), although minute amounts of floridoside and isofloridoside have been reported in *C. merolae* (Nagashima and Fukuda, 1983). Since the enzymes involved in floridoside biosynthesis (floridoside phosphate synthase and floridoside phosphate dephosphorylase; Meng and Srivastava, 1993) are not known at the molecular level, we cannot unequivocally answer the question whether *C. merolae* is able to synthesize floridosides. Floridoside biosynthesis involves the transfer of a galactosyl-unit from UDP-Gal onto glycerol-3-P by floridoside phosphate synthase. In contrast to *G. sulphuraria*, the *C. merolae* genome does not encode any proteins with similarities to known galactosyl-transferases, and one of the putative galactosyl-transferase-like proteins encoded by the Galdieria genome could be a floridoside phosphate synthase (Fig. 1). The successive step in floridoside biosynthesis is the dephosphorylation of floridoside-phosphate by floridoside phosphate dephosphorylase. The Suc-P phosphatase-related protein found only in the *G. sulphuraria* genome could harbor floridoside-phosphate phosphatase activity.

DISCUSSION

In this report, we describe an initial comparison of the genomes of the unicellular thermo-acidophilic red algae *C. merolae* and *G. sulphuraria*. Although the Galdieria dataset is currently limited to some 3,000 ESTs and approximately 8 Mb of genomic contigs (approximately 70% genome coverage), the emerging picture already allows a number of important conclusions: (1) Despite their evolutionary distance, the Cyanidiales have retained a high level of overall similarity in their genomes; (2) Galdieria genes contain more introns; (3) only Galdieria is capable of metabolizing complex cell wall polysaccharides; (4) the lack of heterotrophy in Cyanidioschyzon is not accompanied by a strong reduction in its carbohydrate metabolism enzymatic makeup; and (5) Galdieria harbors many more membrane transporters than Cyanidioschyzon.

Galdieria Has More Carbohydrate Transporters Than Cyanidioschyzon

The metabolic flexibility of *G. sulphuraria* in terms of carbohydrate use is clearly reflected by the many distinct carbohydrate transporters encoded by its genome. We have found 28 genes encoding putative carbohydrate transporters and 3 genes encoding putative glycerol permeases, whereas the genome of *C. merolae* encodes only a single putative monosaccharide transporter. This finding nicely corroborates previous physiological studies demonstrating that a broad range of sugars and sugar alcohols can be taken up by a large number of transporters in Galdieria cells (Oesterhelt et al., 1999; Oesterhelt and Gross, 2002) and subsequently be used as carbon sources for heterotrophic growth (Gross and Schnarrenberger, 1995a).

The enzymatic makeup of the central carbohydrate metabolism of Galdieria and Cyanidioschyzon appears very similar, especially with respect to sugar kinases and polyol dehydrogenases that are present in both genomes, despite the obligatory photoautotrophic nature of *C. merolae*. Previous studies on the central cell metabolism of *G. sulphuraria* gave no indication for a strong regulation of enzyme activity under auto- and heterotrophic conditions (Oesterhelt et al., 1996; Heilmann et al., 1997; Stein et al., 1997). *C. merolae's* inability of heterotrophic growth is therefore most likely not due to the loss of a regulatory machinery but to the lack of suitable solute transport systems.

Cyanidiales Genomes Do Not Encode Plastidic Dicarboxylate Transporters

Surprisingly, neither of the genomes of both algae seems to encode plastidic dicarboxylate translocators similar to those in green plants. In green plants, these transporters are required for nitrogen assimilation and are essential in the photorespiratory pathway (Weber et al., 1995, 2004; Weber and Flügge, 2002; Renné et al.,

2003; Weber, 2004). Both Galdieria and Cyanidioschyzon encode Gln synthetase (GS) on the nuclear genome, whereas Glu synthase (GOGAT) is encoded on the plastid genome in *C. merolae* (Ohta et al., 2003) and *C. caldarium* RK1 (Glöckner et al., 2000). From our genome analysis, it is unclear how the plastidic GS/GOGAT pathway is provided with the precursor 2-oxoglutarate and how the end product, Glu, is exported to the cytosol. Obviously, the plastidic two-translocator system for 2-oxoglutarate and Glu (Weber and Flügge, 2002) does not operate in these red algae, and alternative yet unknown pathways or transporters have to be postulated. An alternative pathway for ammonia assimilation that is not dependent on the GS/GOGAT cycle exists in the unicellular green alga *Chlorella sorokiniana*. In this alga, the alternative splicing of the precursor-mRNA encoding NADP-specific Glu dehydrogenase yields two distinct isozymes of NADP-specific Glu dehydrogenase that have very different ammonium affinities (Miller et al., 1998).

Polyglucan Metabolism

Glucan metabolism as encoded by the Galdieria genome exhibits a higher degree of diversity than that of *C. merolae*. Key differences are the lack of an indirect debranching enzyme (with a putative double activity of α-1,4-glucanotransferase and amylo-(1,6)-glucosidase) and amylopullalanase [for the hydrolysis of both α-(1,6)- and α-(1,4)-glycosidic linkages] in the latter. The lack of the indirect debranching enzyme in *C. merolae* has also been reported by Coppin et al. (2005). This lower degree of enzymatic flexibility in the alga, however, does not result in major structural differences of the polyglucans since the basic enzymatic activities for linking and breaking of α-(1,4)- and α-(1,6)-glucosides are encoded in both genomes (e.g. isoamylase, α and β amylase, branching enzyme, D-enzyme). This is in agreement with earlier studies of debranching activity-deficient mutants of maize, rice, and Chlamydomonas, which were unable to synthesize highly branched granular starch but accumulated phytoglycogen instead (Nakamura et al., 1997; Wang et al., 1998; Zeeman et al., 1998; Kubo et al., 1999; Dauvillée et al., 2000; Hussain et al., 2003). Coppin et al. (2005) suggested that the production of semicrystalline polysaccharides is dependent on the activity of isoamylase and the glucan-water-dikinase R1, both mainly involved in the degradation of starch.

The fact that only a few enzymes with little redundancy are involved in glucan metabolism of *C. merolae* is advantageous for the study of polysaccharide metabolism. Targeted gene knockout should soon be established in Cyanidioschyzon (Minoda et al., 2004); this would make the Cyanidiales suitable objects for genetic analysis of starch metabolism. As recently pointed out, a thorough knowledge of this pathway in Rhodophyta is of crucial interest to understand the evolution of polyglucan biosynthesis following secondary endosymbiosis (Coppin et al., 2005).

Osmotic Adaptation and the Split of the Cyanidiales into Microhabitats

The three Cyanidiales, Galdieria, Cyanidium, and Cyanidioschyzon, occupy the same acidic habitats, and *G. sulphuraria* grows mainly endolithically at these sites (Brock, 1978; Gross et al., 1998; Gross and Oesterhelt, 1999). A recent environmental sampling study demonstrated that Galdieria species preferentially colonized drier areas of the habitat, whereas Cyanidioschyzon was absent from relatively dry, endolithic sites but was abundant in more humid niches of the microenvironment (Ciniglia et al., 2004). The limitation of *C. merolae* to osmotically stable habitats can be explained by a reduced capacity for osmoadaptation in this alga. As corroborated by our molecular data, this is likely due to the absence of (1) a cell wall, (2) synthesis of osmolytes such as floridosides, (3) and a lower degree of cellular compartmentation as indicated by the absence of a vacuole and a limited set of membrane transporters.

Floridoside and isofloridoside represent the major soluble carbohydrate in many red algae (Kremer and Kirst, 1981; Karsten et al., 1993, 1999; Li et al., 2001) and thus have a similar role as Suc in green plants. The occurrence of floridoside has been demonstrated for Galdieria and Cyanidium; its occurrence in Cyanidioschyzon, however, is questionable (De Luca and Moretti, 1983; Nagashima and Fukuda, 1983). Although the pathway for floridoside biosynthesis has been unraveled at the biochemical level (Kremer and Kirst, 1981), genes encoding the corresponding enzymes are not known. Our comparison of the genome sequence of *C. merolae* with genomic sequence reads and ESTs from *G. sulphuraria* revealed several candidate genes that are potentially involved in this biochemical pathway (Fig. 1; Table I). Several of them are confined to the floridoside-synthesizing alga (galactosyltransferase, Suc-P phosphatase), and others can be found in both genomes (Suc-P synthase, trehalose-P synthase, trehalose phosphatase). The ones missing from *C. merolae* represent the most promising candidate genes potentially involved in floridoside biosynthesis.

Recently, floridoside has been suggested as a direct precursor of polysaccharides in the cell wall of *Porphyridium* sp. (Li et al., 2002). As outlined above, a major structural difference between *G. sulphuraria* and *C. merolae* is the absence of a cell wall from the latter alga. The genome of *G. sulphuraria* encodes substantially more putative fucosyltransferases, galactosyltransferases, xylanases, and nucleoside-diphospho-sugar-epimerases than the *C. merolae* genome, and many of these enzymes might be involved in cell wall metabolism. Alternatively, the lack of a cell wall in *C. merolae* might also be due to a mutation in a single gene, as is has been described for several cell wall-less mutants of *C. reinhardtii* (Davies and Plaskitt, 1971). However, the survival of cell wall-less Chlamydomonas mutants is dependent on the presence of

a contractile vacuole that is able to maintain the osmotic potential of the cytosol in hypoosmotic media by pumping water to the extracellular space (Luykx et al., 1997a, 1997b; Allen and Naitoh, 2002). Hence, a mutation causing a defect in cell wall biosynthesis in Chlamydomonas can be compensated by the presence of a contractile vacuole. Such contractile vacuoles have not been described in Cyanidiales, and a single mutational event leading to the absence of a cell wall is thus expected to severely impair the ecological fitness of the corresponding mutant.

In addition to its more flexible carbohydrate metabolism, Galdieria also shows a higher degree of intracellular compartmentalization (e.g. presence of a vacuole) in comparison to Cyanidioschyzon (Albertano et al., 2000). Although our dataset does not allow predictions of the subcellular localization of soluble and membrane proteins, it is likely that some of the membrane transporters detected in our study reside in the tonoplast membrane. Plant vacuoles are important organelles for maintaining and regulating turgor pressure and the osmotic potential of plant cells. Hence, the presence of a central vacuole in Galdieria cells might be crucial to its adaptation to relatively dry parts of hot, acidic habitats because it may help to stabilize the osmotic potential of the cytosol. Notably, the presence of a vacuole coincides with the presence of a rigid cell wall in Galdieria, whereas both are absent from Cyanidioschyzon.

A likely scenario for the splitting of the Cyanidiales into microenvironments is therefore an evolution from a common, cell wall-bearing ancestor. *C. merolae* has adapted to osmotically stable niches within the hot and acidic habitat, and this specialization might have led to a gradual loss of genes involved in the regulation of the osmotic homeostasis, such as those genes involved in the biogenesis of a cell wall and a vacuole and in the biosynthesis of compatible solutes such as floridoside.

The microhabitat of the third member of the Cyanidiales, *C. caldarium*, is less well characterized, most likely due to the difficulty of isolating this alga from field samples. It can be speculated, though, that due to the presence of a cell wall, a central vacuole, and the osmolyte floridoside, *C. caldarium* should colonize similar niches as *G. sulphuraria*. However, light measurements inside stones have shown that autotrophic growth at endolithic sites is possible only within the first few millimeters beneath the surface layer (Gross and Oesterhelt, 1999). The inability of *C. caldarium* to grow heterotrophically thus limits potential for endolithic cell growth significantly. Additional field and molecular studies will be required to further characterize the microhabitat of Cyanidium.

CONCLUSIONS AND PERSPECTIVES

Comparative genomics is a powerful tool to unravel previously unknown gene functions. The genomes of the closely related but physiologically very different species Galdieria and Cyanidioschyzon are particularly interesting objects for a comparative approach. The Cyanidiales have been established as a monophyletic sister group to all other red algae and are sister to the lineage that gave rise, through secondary endosymbiosis, to the plastid in the Chromista (Yoon et al., 2002, 2004). A thorough understanding of these extremophilic unicells is therefore of significant importance. Although the Galdieria genome sequence has not been finished, this initial comparative analysis of the two genomes has already yielded several interesting testable hypotheses for future research. For example, Galdieria's ability to use glycerol as a carbon source may be dependent on aquaporin-like glycerol permeases in its plasma membrane. In addition, we hypothesize that a Suc-6-P phosphorylase-like gene in Galdieria is required for floridoside biosynthesis and that storage glucan synthesis is initiated by glycogenin-like proteins. To test these hypotheses, we will need to establish transformation and gene knockout protocols for Galdieria to analyze the effects of the corresponding gene knockouts. Such protocols have been developed for Cyanidioschyzon (Minoda et al., 2004) and it should be possible to establish similar protocols for Galdieria, too. Alternatively, the genes of interest can be expressed in recombinant hosts, and the function of the gene products can be tested in vitro. To complete the picture of molecular evolution of Cyanidiales, a genome project for *C. caldarium* would be necessary. The many advantages of unicellular algae as model systems to study plant biology have recently been reviewed (Hicks et al., 2001) and this focus issue of *Plant Physiology* underlines the value of these systems. These model systems will become even more useful tools with the availability of additional algal genome sequences in the near future.

MATERIALS AND METHODS

Strains and Media

Galdieria sulphuraria strain 074W was cultivated axenically in mineral medium supplemented with 25 mM Gal at 37°C in the dark as described previously (Gross and Schnarrenberger, 1995a).

Construction of Genomic DNA Libraries

Cells equivalent to 2 g fresh weight were frozen in liquid nitrogen and ground to a fine powder by mortar and pestle. Nucleic acids were extracted by incubating the ground tissue overnight in extraction buffer (50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% [w/v] SDS), followed by extraction of proteins with phenol:chloroform:isoamylalcohol (24:24:1) and precipitation of DNA from the aqueous phase by ethanol. The pellet was dissolved in Tris-EDTA buffer and RNA was removed by incubation with DNAse-free RNAse, followed by deproteination with phenol:chloroform:isoamylalcohol, and DNA precipitation by ethanol. Genomic DNA was further purified by CsCl-density gradient centrifugation of bis-benzamide-treated total DNA as described previously (Chiu et al., 1990).

The small-insert shotgun sequencing plasmid library was constructed in pSMART-HC Kan (Lucigen, Middleton, WI; www.lucigene.com). Genomic DNA was randomly fragmented by shearing using a HydroShear device (Thorstenson et al., 1998) and end repaired to generate blunt ends. Fragments of approximately 2 kbp (isolated by preparative agarose gel electrophoresis) were ligated into the pSMART vector and plasmids were transformed into *E. cloni* cells (Lucigen).

Isolation of Plasmid DNA, DNA Sequencing, and Data Storage

Colonies were randomly picked using a GeneMachines Mantis Colony and Plaque Picker (GeneMachines, San Carlos, CA), and plasmid DNA was prepared from overnight cultures using a Qiagen 3000 robot (Qiagen USA, Valencia, CA). DNA sequences were determined by cycle sequencing and sequence analysis using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). All sequence data and chromatograms were stored on a Geospiza Finch server (Geospiza, Seattle).

Sequence Data and Similarity Searches

Cyanidioschyzon merolae predicted polypeptide sequences were obtained from http://merolae.biol.s.u-tokyo.ac.jp (Matsuzaki et al., 2004), data released March 16, 2004. Genomic sequences of *G. sulphuraria* were screened against a database consisting of *Porphyra purpurea* chloroplast, *Cyanidium caldarium* str. RK1 chloroplast, and *C. merolae* plastid and mitochondrion sequences as published in GenBank. EST libraries and sequences were reported previously (Weber et al., 2004).

Similarity searching was done with the BLAST 2.2.6 program (NCBI; Altschul et al., 1997). Data were parsed and analyzed using software implementations developed by the Genomic Technology Support Facility at Michigan State University.

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